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Analysis of the antibody response in Atlantic salmon against recombinant VP2 of infectious pancreatic necrosis virus (IPNV)

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An IPNV-specific humoral immune response was demonstrated in Atlantic salmon (*Salmo salar*) pre-smolts and in rabbits immunised with purified *E. coli*-expressed recombinant VP2. In ELISA all anti-rVP2 sera generally showed a stronger reaction with rVP2 than with IPNV, except for one rabbit anti-rVP2 serum that reacted more strongly with IPNV than with rVP2. In Western blotting, both anti-rVP2 and anti-IPNV sera reacted well with viral VP2 and rVP2, but all anti-IPNV sera also reacted with VP3. IPNV-neutralising antibodies were detected in all tested anti-IPNV sera but not in anti-rVP2 sera. However, the anti-rVP2 and anti-IPNV sera had comparable IPNV titre-reduction capacity when immunocomplexes were subtracted from a mix of IPNV and antisera, indicating an antiviral effect *in vivo*.

Key words: Atlantic salmon, recombinant VP2, IPNV, immune response.

I. Introduction

In Norway, infectious pancreatic necrosis virus (IPNV) was first isolated from Atlantic salmon in 1975 (Håstein & Krogsrud, 1976). Several reports in the 1980s indicated that IPNV caused clinical disease with high mortality in farmed Atlantic salmon fry in Norway (Melby *et al.*, 1991). For salmonids, mortality due to IPN is generally considered to by a fry disease limited to fry at ages below 6 months (Frantsi & Savan, 1971). However, in Norway annual outbreaks of IPN among post-smolts of Atlantic salmon have been reported since 1989 (Melby *et al.*, 1994).

IPNV is a well characterised virus belonging to the birnavirus genus, family *Birnaviridae*, and several serotypes are known to be pathogenic to fish (Hill & Way, 1995). The virus genome consists of two RNA segments, A and B, of which A encodes the two known structural proteins, VP2 and VP3 (Duncan & Dobos, 1986; Håvarstein *et al.*, 1990*b*). The major neutralisation epitopes of IPNV are localised within VP2 (Caswell-Reno *et al.*, 1986; Christie *et al.*, 1990; Frost *et al.*, 1995) but neutralisation epitopes have also been suggested for VP3

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(Tarrab *et al.*, 1993, 1995). It has been demonstrated that two variable and one conserved neutralisation epitopes, all conformation-dependent and partly overlapping, are localised within the central third part of VP2 (Frost *et al.*, 1995). Recombinant VP2, with structures that resemble these epitopes, has been a candidate vaccine in a major vaccine development project resulting in a commercial injectable IPNV vaccine that was recently licensed in Norway (Frost & Ness, 1998). Previously, we demonstrated that the commercial vaccine did not induce any measurable humoral immune response post-vaccination although it suppressed viral replication post-challenge (Frost & Ness, 1998). In this study we analysed whether high doses of purified rVP2 could induce a detectable IPNV-specific immune response in Atlantic salmon and in rabbits.

II. Materials and Methods

CELLS AND VIRUS

IPNV (N1) was propagated in Chinook salmon embryo cells (CHSE-214) as described by Christie *et al.* (1988). Virus for enzyme-linked immunosorbent assay (ELISA) and SDS-polyacrylamide gel electrophoresis (PAGE) was propagated using medium without foetal bovine serum. Virus for SDS-PAGE and immunisation was purified by CsCl gradient centrifugation as described by Christie *et al.* (1988).

FISH STOCK AND REARING CONDITIONS

Juvenile Atlantic salmon (*Salmo salar* L.), unvaccinated and licensed by veterinary control as free from furunculosis, bacterial kidney disease, yersiniosis, cold-water vibriosis, infectious salmon anaemia, whirling disease and without any previous history of infectious disease, were used. The fish, which had a mean weight of 20·4 g, were kept in rectangular 200 l tanks with running, UV-treated fresh water and with a constant temperature of 10° C. Pellet feed (Felleskjøpet, Norway) was dispensed from automatic feeders. The fish were starved for 24 h and anaesthetised with Methiomidate before immunisation and sampling.

PURIFICATION OF RECOMBINANT VP2

E. coli BL21 DE3 pLysS pET11d-VP2 were grown and VP2 expression induced as described by Frost *et al.* (1995). The cells were harvested by centrifugation (3000 × *g* for 20 min), concentrated 10 times when re-suspended in ice-cold 1 × TNE-T buffer and the inclusion bodies containing rVP2 were washed as described earlier (Frost *et al.*, 1995). Further rVP2 purification was performed by preparative SDS-PAGE (Prep Cell, BioRad, U.S.A.). A 9 cm 12·5% acrylamide separating gel and a 2 cm 4% stacking gel were polymerised in the 28 mm diameter tube of the preparative gel apparatus. Washed inclusion bodies from a 100 ml culture corresponding to 5–10 mg total protein, were dissolved in 2 ml sample buffer (42 mM Tris, pH 6·8, 8% glycerol, 3% β-mercaptoethanol, 0·1% SDS, 1% bromophenol blue) and incubated at 50° C

for 10 minutes before loading. The gel was run at 70 mA constant current until the bromophenol blue marker dye was about 5 mm from the bottom of the separating gel. The current was reduced to 40 mA constant current and running buffer (50 mM Tris, pH 8·3, 384 mM Glycine, 0·1% SDS) pumped through the elution chamber at a rate of 0·5 ml/min. The elution chamber outlet was connected to a fraction collector and 4·2 ml fractions collected. In order to locate the fractions containing the recombinant protein, every fifth fraction was analysed by Western blotting with a rabbit anti-IPNV serum. The purity of eluted rVP2 was analysed by SDS-PAGE and silver nitrate staining for detection of proteins and LPS (Aakre *et al.*, 1994). The best fractions with respect to rVP2 purity were pooled and concentrated by dehydration using polyethyleneglycol (MW 20 000). The rVP2 concentration was estimated by OD_{280} measurement using an extinction coefficient of E 1 cm=10·0 for 1% solution of recombinant protein and by semi-quantitative SDS-PAGE. In SDS-PAGE the concentration of the rVP2 was estimated by comparison to a known quantity of bovine serum albumin stained with Coomassie blue.

IMMUNISATION AND SAMPLING

During immunisation the fish were divided into two groups of 15 fish and injected intraperitoneally with 0·25 ml Freund's Complete Adjuvant (FCA) added to 0·25 ml rVP2 (2 mg) or Hank's Balanced Salt Solution (HBSS), respectively. Blood samples were collected from the vena cardinalis communis 8 weeks after injection.

Salmon anti-IPNV was produced by mixing sera from 107 individual fish challenged with IPNV by injection.

The rabbits were immunised twice, 8 weeks apart, with 0·1 ml PBS (controls) or 200 μg recombinant VP2 in 0·1 ml PBS, both mixed with FCA (1:1 vol/vol). The first dose was injected intramuscularly and the second subcutaneously. Blood was sampled pre-immunisation and 4 weeks after the second injection. Rabbit antisera against IPNV were produced as described earlier (Christie *et al.*, 1988). Blood was allowed to clot at 4° C for 12 h, sera harvested and stored in aliquots at − 80° C until used.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA using IPNV as antigen was performed as described elsewhere (Håvarstein *et al.*, 1990*a*). Virus supernatant (0·1 ml of 10^7 $TCID_{50}$/ml) or 0·1 ml of rVP2 (4 μg/ml) were used to coat individual wells (Immunoplates, Nunc Maxi Sorb, Denmark). The coated plates were incubated at 4° C for 18 h and post-coated with 5% fat-free dry milk (Nestlé) in phosphate-buffered saline (PBS) containing 0·05% Tween-20 (PBS-Tween) for 1 h at room temperature. All subsequent incubations were carried out in PBS-Tween containing 1% fat-free dried milk, and PBS-Tween was used for washing between incubations. Salmon antisera were diluted two-fold and incubated for 4 h at 15° C. Rabbit anti-salmon Ig were diluted 1:3000 as described earlier (Håvarstein *et al.*, 1990*a*). Rabbit antisera were diluted two-fold and incubated for 1 h at room temperature. Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit-IgG (Bio-Rad, U.S.A.) diluted 1:3000 was incubated for 1 h at room

temperature. O-Phenylenediamine dihydrochloride (Sigma), 100 μl per well, was added as substrate and colour development stopped after 20 min by addition of 50 μl H_2SO_4 per well. Plates were read spectrophotometrically at 492 nm using a Titertek ELISA reader.

SDS PAGE AND WESTERN BLOTTING

Electrophoresis was performed using a Mini Protean II Dual Slab Cell system (Bio-Rad, U.S.A.) as described earlier (Håvarstein *et al.*, 1990*a*). For Western blotting analysis, proteins were transferred to a nitrocellulose membrane (Towbin *et al.*, 1979) using 100 V for 1 h. After transfer the membranes were blocked with a 3% non-fat dry milk (Nestlé) for 1 h at 4° C. Salmon antisera (1:100) and rabbit-antisera (1:200) were incubated for 16 h at 4° C and the membrane washed three times in PBS-T. Salmon antibodies were detected using rabbit anti-salmon Ig serum (1:1000), incubated for 1 h at room temperature. HRP-conjugated goat anti-rabbit-IgG (Bio-Rad, U.S.A.) was diluted 1:3000 and incubated for 1 h at room temperature. Colour development with HRP colour development reagent (Bio-Rad, U.S.A.) was performed for 30 min.

NEUTRALISATION ASSAY

Sera from rabbits and salmon were diluted two-fold from 1:100 to 1:1600, mixed with an equal volume of an IPNV suspension (10^3 $TCID_{50}$/ml) and incubated for 1 h at room temperature. Samples (0·1 ml) were added to individual wells in 96-well tissue culture plates containing CHSE-214 cell monolayers. The cell cultures were examined for cytopathic effect (CPE) at day 3, 7 and 12. Positive (only virus) and negative (only antiserum) controls were used on all plates.

ABSORPTION OF IPNV-ANTIBODY COMPLEXES WITH SEPHAROSE

Salmon (50 μl) or rabbit (10 μl) antisera were mixed with 100 μl IPNV suspension (10^7 $TCID_{50}$/ml) and incubated for 2 h with gentle mixing every 15 min. Rabbit anti-salmon Ig linked to Sepharose or Sepharose-protein G (Pharmacia Biotec) was added to the tubes with salmon or rabbit sera, respectively, and the volume adjusted to 1 ml using PBS. After 1 h the tubes were centrifuged (300 × *g*, 10 min) to remove the Sepharose. The supernatants were diluted to samples with $TCID_{50}$/ml titre of 10^7, 5×10^6, 10^6, 5×10^5, 10^5, 5×10^4, 10^4, 5×10^3, 10^3, 5×10^2, 10^2, and 0·1 ml of each sample transferred to individual wells in a 96-well tissue culture plate containing CHSE-214 monolayer. The cell cultures were examined for CPE as described for the neutralisation assay and the remaining $TCID_{50}$ (Reed & Muench, 1938) estimated.

III. Results

In salmon immunised with rVP2, a strong humoral immune response against rVP2 (Fig. 1a) and a moderate response against IPNV (Fig. 1b), were detected

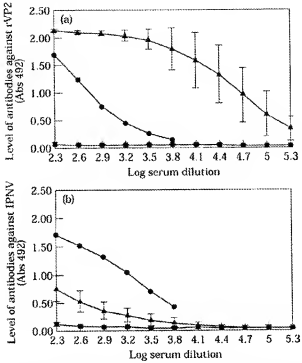

Fig. 1. Level of antibodies against rVP2 (A) and IPNV (B) in sera from Atlantic salmon (n=15) immunised with rVP2 in FCA (—▲—) and in a pooled (n=107) antiserum from Atlantic salmon challenged with IPNV (—●—). Saline injected control (—■—).

in ELISA. All the salmon anti-rVP2 sera recognised rVP2 and IPNV VP2 in Western blot (data not shown) while none of the tested control sera showed any reaction. The pooled salmon anti-IPNV serum from fish challenged with IPNV showed a good reaction against both rVP2 and IPNV in ELISA. In Western blots, the salmon anti-IPNV serum also recognised VP3.

In ELISA, one of the rabbit anti rVP2 sera (ø48) reacted strongly with rVP2 (Fig. 2a) and more weakly with IPNV (Fig. 2b). The other rabbit anti-rVP2 serum (ø49) showed better reactivity with IPNV than with rVP2. Both rabbit anti-IPNV sera (ø456 and ø457) reacted strongly with both rVP2 (Fig. 2a) and IPNV (Fig. 2b). In Western blotting the four rabbit antisera all reacted well with both rVP2 and IPNV VP2 (data not shown), but two rabbit anti-IPNV sera also reacted with VP3.

None of the anti-rVP2 sera were positive in the virus neutralisation assay but the pooled antisera from salmon challenged with IPNV and both rabbit anti-IPNV sera neutralised the virus (10^2 $TCID_{50}$) diluted up to 400 × and above 1000 ×, respectively. The salmon anti-live IPNV serum and 4 out of 5 tested salmon anti-rVP2 sera reduced the IPNV titre 10-fold from 10^7 to 10^6 $TCID_{50}$/ml when coupled to Sepharose (Table 1). The tested rabbit anti-rVP2 (ø48) and anti-IPNV (ø456) both reduced the IPNV titre from 10^7 to 10^4 $TCID_{50}$/ml.

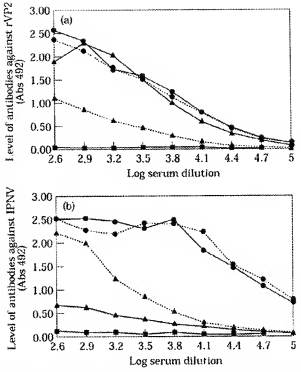

Fig. 2. Level of antibodies against rVP2 (A) and IPNV (B) in rabbit preserum (mean of two —■—) and sera from rabbits immunised with rVP2 in FCA (ø48=—▲— and ø49=- - -▲- - -) or IPNV (— ●— and - - -●- - -).

IV. Discussion

These results demonstrate that purified *E. coli*-expressed rVP2 of IPNV induces production of IPNV-specific antibodies in Atlantic salmon and in rabbits. Antisera against rVP2 generally showed a moderate reaction with IPNV in ELISA while antisera against IPNV reacted well with rVP2. However, in Western blotting the anti-IPNV and the anti-rVP2 sera showed a similar reaction pattern. This indicates that most epitopes exposed on IPNV are either not exposed on rVP2, exposed on rVP2 in a non-immunogenic form or with a structure too different from viral epitopes to induce antibodies with the correct paratope. However, one of the rabbit-anti-rVP2 sera contained low levels of rVP2-specific antibodies but high levels of IPNV-specific antibodies indicating that, at least in rabbits, some correctly folded B-cell epitopes can be recognised, either on the rVP2 surface (direct B-cell stimulation) or following degradation and exposure by antigen presenting cells.

In our experience the rVP2 dose is of major importance for induction of a detectable humoral immune response. In some earlier experiments we immunised salmon with 1 and 10 μg rVP2/g fish with only a very low homologous response and no IPNV-specific response (unpublished). The high antigen dose needed to induce a good response in salmon (10–100 μg/g fish) indicate that the immunogenicity of rVP2 with respect to the humoral immune response is low.

Table 1. IPNV titre reduction after absorption of immune-complexes between antisera and IPNV using Sepharose-protein G (rabbit sera) or Sepharose-rabbit-anti-salmon-Ig (salmon sera)

Antisera added to IPNV	IPNV titre reduction after Sepharose absorption
Rabbit anti-rVP2 (ø48)	1000-fold ($10^7 \rightarrow 10^4$)
Rabbit-anti-IPNV (ø456)	1000-fold ($10^7 \rightarrow 10^4$)
Rabbit preserum (mean of two)	0 ($10^7 \rightarrow 10^7$)
Salmon anti-rVP2 (1)	10-fold ($10^7 \rightarrow 10^6$)
Salmon anti-rVP2 (2)	10-fold ($10^7 \rightarrow 10^6$)
Salmon anti-rVP2 (3)	10-fold ($10^7 \rightarrow 10^6$)
Salmon anti-rVP2 (4)	10-fold ($10^7 \rightarrow 10^6$)
Salmon anti-rVP2 (5)	0 ($10^7 \rightarrow 10^7$)
Salmon anti-saline (1)	0 ($10^7 \rightarrow 10^7$)
Salmon anti-saline (2)	0 ($10^7 \rightarrow 10^7$)
Salmon anti-saline (3)	0 ($10^7 \rightarrow 10^7$)
Salmon anti-saline (4)	0 ($10^7 \rightarrow 10^7$)
Salmon anti-saline (5)	0 ($10^7 \rightarrow 10^7$)
Salmon anti-live-IPNV (pooled)	10-fold ($10^7 \rightarrow 10^6$)

This is supported by the fact that in rabbits IPNV induces a stronger response against rVP2 than rVP2 itself, even when the rVP2 dose is larger.

Although the anti-rVP2 sera were non-neutralising and had low titre with respect to IPNV detection, the salmon anti-rVP2 sera recognised epitopes on virus in solution and were able to reduce the virus titre 10-fold in an immunecomplex subtraction assay, comparable to what was obtained using anti-IPNV serum.

The importance of antibodies in a protective immune response against IPNV is unknown. For trout it has been demonstrated that immunity against IPNV can be transferred with serum (Agneil, 1975) indicating that the humoral immune response is an important factor in the defence against IPNV. Furthermore, a correlation between virus clearance and the level of neutralising antibodies in rainbow trout has been demonstrated (Yamamoto, 1975), but evidence from other salmonid species has revealed no correlation (Reno, 1976; Smail & Munro, 1985). Bootland *et al.* (1995) immunised adult brook trout with a high dose of inactivated IPNV which induced a high humoral immune response with IPNV-neutralising antibodies. However, the antibodies did not prevent infection following challenge. Previously, we have demonstrated that vaccination of salmon with a multivalent vaccine containing rVP2 suppresses IPNV replication post-challenge although no IPNV-specific antibodies could be detected pre-challenge (Frost & Ness, 1998). However, after challenge the production of IPNV-specific antibodies were higher and faster than in control groups, indicating that an undetectable IPNV-specific B-cell response was induced after vaccination, as is supported by this study.

Many neutralisation epitopes have been reported for VP2 of aquatic birnaviruses by production of monoclonal antibodies (Caswell-Reno *et al.*, 1986; Christie *et al.*, 1990; Frost *et al.*, 1995; Tarrab *et al.*, 1995). Most of the

epitopes have been regarded as important in protective immunity including those exposed on rVP2 (Frost *et al.*, 1995). However, it is not known whether epitopes on IPNV, recognised by virus-neutralising monoclonal antibodies produced in mice, induce comparable neutralising antibodies in salmon. For mammals, cellular attack on infected host cells prior to viral shedding is considered the most important anti-viral defence (Whitton & Oldstone, 1996) and it is generally accepted that specific antibodies against a virus, even if virus-neutralising *in vitro*, do not necessarily protect against viral disease. Although the importance of IPNV-specific antibodies in immunity against IPN is unknown, they indicate a specific immune response. The level and complexity of the IPNV specific immune response induced by rVP2 in this study is unknown. However, the B-cells with IPNV-specific antibodies can probably quickly produce antibodies (Frost & Ness, 1998) able to opsonise IPNV-infected cells and free virus particles. This can stimulate macrophage activity, induce cytokine production and activate a protective cellular immune response. Most likely, rVP2 expressed in a eukaryotic vector system like yeast or insect cells (Magyar & Dobos, 1994) would, like inactivated IPNV (Bootland *et al.*, 1995), be a better antigen with respect to a humoral immune response as demonstrated for VHSV (Lecocq-Xhonneux *et al.*, 1994). However, the lack of cost-efficient large scale production of low cost vaccines limits most eukaryotic expression systems to scientific use. To be able to develop new, cheap and effective vaccines for the growing aquaculture industry more research should focus on the immune system of aquatic organisms, especially the cellular immune system.

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